

Award Number: **W81XWH-07-1-0044**

TITLE: **Identification of the Transformational Properties and
Transcriptional Targets of the Oncogenic SRY Transcription Factor SOX4**

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REPORT DATE: **January 2009**

TYPE OF REPORT: **Annual** Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 01-10-2009		2. REPORT TYPE Annual Summary		3.DATES COVERED: 11 DEC 07- 10 DEC 08	
4. TITLE AND SUBTITLE: Identification of the Transformational Properties and Transcriptional Targets of the Oncogenic SRY Transcription Factor SOX4				5a. CONTRACT NUMBER W81XWH-07-1-0044	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Christopher Scharer				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Emory University Holly Sommers 1784 N Decatur Rd, Suite 530 Atlanta, GA 30322				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5102				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT: Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT SOX4 is a critical developmental transcription factor in vertebrates and is required for precise proliferation and differentiation in multiple tissues. SOX4 has also been implicated in tumorigenesis of multiple tumor types and has been shown by our lab to be upregulated in prostate cancer. However, the exact molecular role and the genes SOX4 transcriptionally influences remain unknown. Using chromatin immunoprecipitation coupled to DNA microarrays (ChIP-chip) we have identified 282 high confidence SOX4 target genes and 3600 genomic binding sites. We have also used a unique protein binding, double-stranded DNA microarray to determine a novel SOX4 position-weight matrix for <i>in silico</i> binding site searches. Direct targets of SOX4 include key cellular regulators such as EGFR, Tenascin C, Frizzled-5 and Patched-1. In addition, SOX4 impacts other transcriptional networks through regulation of 23 transcription factors. We also show that SOX4 impacts the microRNA-processing pathway through direct regulation of Dicer and Argonaute 1. For the first time, these data provide a snapshot of the global transcriptional regulatory network of SOX4 in prostate cancer.					
15. SUBJECT TERMS ChIP-chip, SOX4, Prostate Cancer, Protein-binding microarray					
16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 27	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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Introduction:

SOX4 is a critical developmental transcription factor and is required for precise differentiation and proliferation in multiple tissues. SOX4 is a 47-kDa protein that is encoded by a single exon and contains a conserved high mobility group (HMG) DNA-binding domain (DBD) related to the TCF/LEF family of transcription factors. Our lab has previously shown SOX4 mRNA and protein to be overexpressed in prostate cancer, and this expression is correlated with increasing Gleason score. Other labs have shown SOX4 mRNA to be overexpressed in other tumors such as leukemia, melanoma, glioblastoma and bladder carcinomas. However, despite this knowledge little is known of the direct transcriptional targets of SOX4, and how misregulation of these networks affects human cancers and development. The goal of this research is to determine the transcriptional target genes of SOX4 and to determine SOX4's role in murine prostate development. To determine the direct transcriptional targets on a global scale we performed chromatin immunoprecipitation coupled to DNA microarrays. We used human promoter arrays from NimbleGen, Inc. that tiled roughly 5 kb of promoter and intronic sequence for 25,000 known transcripts. In total the array tiled 110 Mb of DNA. Using this technique we were able to determine the genes with SOX4 bound at their promoter in living prostate cancer cells. Furthermore, expression profiling of prostate cancer cells overexpressing either SOX4 or a control vector identified those genes that are transcriptionally regulated by SOX4. We have also obtained a SOX4 floxed mouse that will enable the prostate specific deletion of SOX4 in mice. This information will determine if SOX4 is required for the development of a functional prostate. Determining the transcriptional targets and *in vivo* functions of SOX4 will contribute critical knowledge to the SOX4 field and further our understanding of SOX4's role in development and carcinogenesis.

Body:

AIM 1: Determine the Direct Transcriptional Targets of SOX4 on a Global Scale using a ChIP-chip and microarray approach.

Chromatin immunoprecipitation (ChIP) relies on high quality, specific antibodies which can immunoprecipitate the protein of interest with little background. While commercial antibodies that recognize SOX4 in immunoblotting applications exist, none have shown activity in immunoprecipitations in our hands. Therefore, an HA epitope tag was introduced onto the N-terminus of SOX4 and cloned into a lentiviral vector for stable infection of mammalian cells (Fig. 1A). The lentivirus contains an eYFP gene to enable the purification by Fluorescent Activated Cell Sorting (FACS) of stably infected cells. For both the LNCaP and RWPE-1 prostate cancer cell lines, both a control eYFP only and an HA-SOX4-eYFP cell line were created and infected cells FACS purified (Fig. 1B). Both cell lines were tested for transgene expression and to ensure HA-SOX4 could be immunoprecipitated using our 12CA5, anti-HA monoclonal antibody (Fig. 1C). ChIP assays were performed from the LNCaP-HA-SOX4 cells in triplicate and in duplicate from the LNCaP-YFP cells. DNA was extracted and purified according to published protocols, and amplified using a Ligation-mediated PCR approach (8). 4 ug of immunoprecipitated and total input DNA was sent to NimbleGen and hybridized to their 25K dual chip Promoter array. The array tiles roughly 5 Kb of promoter and intronic sequence for 25,000 known transcripts with a total coverage of 110 Mb.

Signal intensities were Z-score normalized, log2 transformed and ratios of immunoprecipitated to total input signal calculated for each probe set. ChIPOTle software (2) was used with a 500 bp sliding window to look for sets of neighboring probes that are enriched together. Peaks that overlapped in 2 of the three data sets and were not present in the control data set were identified and called SOX4 binding sites (Fig. 2A). This analysis identified 3,600 binding sites in the promoters of 3,470 different genes. 28 peaks were chosen and 10 verified by ChIP-quantitative Real-Time PCR (qPCR) and 18 by traditional ChIP-PCR. 24 of 28 sites (86%) chosen were specifically enriched in the LNCaP-HA-SOX4 cells and not in the control LNCaP-YFP cells (Fig. 2B and 2C). All validated peaks were also validated in the RWPE-1 cell line except ANKRD15, further validating the data set (Fig. 2C).

While SOX4 binds to the promoters of 3,470 different genes we do not know how SOX4 influences transcription of each gene. To identify genes whose expression changes when SOX4 levels are altered we performed whole genome expression profiling of LNCaP cells transfected with either control vector or HA-SOX4. In order to enrich for direct SOX4 targets, total RNA was harvested 24 hours post-transfection and profiled using an Illumina Human 6-v2 whole genome array. Compared to vector control, 1,766 genes were altered at least 1.5 fold when SOX4 was overexpressed in LNCaP cells (Fig. 3A). Ten of these genes were confirmed by qPCR (Fig. 3B) and SOX4's induction of DICER1 was confirmed at the protein level (Fig. 3C). Previous expression profiling of LNCaP cells transfected with either control siRNA or SOX4 siRNA identified 465 down stream target genes for SOX4 (7). Combining these three data sets we identified 282 genes that had SOX4 bound to their promoter regions and were transcriptionally altered when SOX4 levels were perturbed (Fig. 3D). 9 genes overlapped in all three data sets (PIK4CA, DHX9, BTN3A3, CDK2, MVK, ADAM10, RYK, ISG20, and DBI). Although only 10% of the significant differentially expressed genes overlapped with the ChIP-chip data, this is likely a conservative estimate because the NimbleGen 25K promoter array only queries proximal promoter sequences. Thus, more of the 1,900 genes that responded to changes in SOX4 mRNA levels (but were not detected by ChIP-chip) could still be direct targets. Excellent candidates would be the 40 genes that responded to SOX4 on both microarray platforms, such as the IL6 receptor, SOX12, and NME1. Alternate methods such as ChIP-SEQ would provide a truly unbiased, genomic picture of SOX4 binding. Nevertheless, this is the first

global study of SOX4 binding and provides a foundation for understanding the SOX4 transcriptional network in prostate cancer.

HMG domain transcription factors bind AT rich DNA in the minor groove and two previous reports identify a 7mer SOX4 binding motif (15, 16). While this knowledge can aid in the search for putative binding sites it does not take into account the role of alternate bases at various positions. A SOX4 specific position-weight matrix is required to fully utilize the power of bioinformatic searches. Apart from the consensus core SOX family binding site WWCAAW, where W represents either A or T, little is known about what preferences SOX4 exhibits at each base position during binding (6). In order to facilitate bioinformatic searches for SOX4 DNA binding sites we sought to determine a SOX4 specific position-weight matrix (PWM) using a unique, protein-binding, double stranded DNA microarray (1). The array allows recombinant protein to interact with and bind, every possible 10mer, thus allowing *in vitro* binding site specificities to be calculated. We generated an N-terminal, GST-SOX4-DBD fusion protein, and expressed and purified it from *E. coli* (Fig. 4B). To ensure the purified recombinant fusion protein was functional we performed an electromobility shift assay (EMSA) using a published SOX4 binding site of AACAAAG (15). Increasing concentrations of GST-SOX4-DBD was incubated with radiolabeled specific probe alone, with a cold specific competitor or a cold non-specific competitor. GST-SOX4-DBD was able to bind the probe and cause a shift that was abolished when cold specific competitor probe, but not when cold non-specific probe was added (Fig. 4A). These data show that the truncated GST-SOX4-DBD fusion protein is functionally active *in vitro*. The GST-SOX4-DBD was incubated with the protein binding microarray and a novel PWM (RWYAAWRV) (R – A or G, Y – C or T, and V – G, A or C) was calculated according to published protocols (Fig. 4C) (1). Two groups have previously reported similar binding site sequences for SOX4: AACAAAG (15) and AACAAAT (16). Our PWM confirms both of the previous known binding sites and adds new information on the binding preferences in the 8th position as well as alternate bases at the 6th and 7th positions.

Using our newly determined PWM we sought to establish if the peaks in the promoters of our SOX4 target genes are enriched for SOX4 binding sites. We applied CONFAC software (5) and analyzed the peaks in our 282 high-confidence target genes as well as 10 sets of random control promoter sequence. Control peaks of equal size were selected from at random from promoter sequences covered on the NimbleGen array and each control set represents equal sequence coverage as our 282 high-confidence peaks. With stringent criteria (core similarity: >0.85; matrix similarity: >0.75) we find that 60% of our high-confidence peaks contain SOX4 binding sites. SOX4 sites were significantly enriched compared to our 10 random control sets by Mann-Whitney U test with Benjamini correction for multiple hypothesis testing ($q < 0.0019$). To further characterize the data set we searched each of the 3,600 SOX4 binding sites and 10 sets of control peaks (assembled in the same manner as above) for the presence of Protein-binding Microarray (PBM) bound k-mers. These k-mers are the individual, ungapped 8mer sequences SOX4 bound on the PBM. The specificity of PBM k-mers can be defined by the enrichment score (ES), which ranges from -0.5 to 0.5 (10). We analyzed the enrichment of PBM k-mers with $0.45 > ES > 0.40$ (moderate) and $ES > 0.45$ (stringent). Both SOX4-bound peaks and control peaks contained stringent and moderate k-mers, SOX4 bound peaks contained significantly more stringent ($p = 0.0002$) and moderate ($p = 1.08 \times 10^{-5}$) k-mers by two-tailed Mann-Whitney test. SOX transcription factors have been reported to mediate their transcriptional activity through interactions with other transcription factors such as the SOX2-OCT3/4 pair (6). We applied CONFAC software to search for the presence of co-occurring binding sites enriched in our SOX4 peaks. Interestingly, the E2F family was the most frequently co-occurring motif (Table 1) and Ingenuity Pathway Assist¹ identified cell-cycle as a functionally enriched process in the 3,470 SOX4 target genes. This suggests that part of SOX4's function is to regulate genes involved in the cell-cycle progression. CONFAC also identified co-occurring binding sites for transcription factors involved in the TGF β , WNT, and NF- κ B pathways (Table 1). The presence of

¹ <http://www.ingenuity.com>

WNT pathway transcription factors was particularly interesting considering a previous report that SOX4 co-operates with TCF4 and β -catenin to alter transcription (14). We confirmed this finding in LNCaP cells and found that SOX4 co-operates with β -catenin to increase transcription of a WNT reporter construct (Fig. 5).

In order to determine the biological processes and pathways enriched in SOX4 target genes we performed GO ontology analysis using DAVID software (3). As expected, the top annotated process was transcription ($p = 3.17 \times 10^{-18}$) but surprisingly we also find transmembrane (5.59×10^{-10}) and protein phosphorylation/dephosphorylation ($3.5 \times 10^{-18}/6.6 \times 10^{-7}$) as enriched functions. DAVID software also identified 23 transcription factors as direct SOX4 target genes (Table 2). These data suggest that SOX4 modulates signaling networks at all three cellular levels: at the membrane, in the cytoplasm and inside the nucleus. IPA analysis identified biological pathways enriched in both the 3,470 direct target genes and the 1,766 genes altered when SOX4 is overexpressed. As expected the top annotations were cancer, cell-cycle and tissue development and SOX4 target genes were found to influence wide variety of developmental signaling pathways such as WNT, NOTCH, WNT- β -catenin, PI3K-AKT and the EGFR signaling network. Interestingly, microRNA processing enzymes DICER, AGO1 and the RNA helicase DHX9 were both direct target genes and showed expression changes when SOX4 was overexpressed. For the first time we report a link between a SOX family member and the microRNA processing pathway. Key SOX4 target genes and their cellular localization are illustrated in Figure 6A and 6B.

For a detailed discussion of these results see Appendix II and (13).

AIM2: Determine the effects of Loss or Overexpression in vivo

SOX4 is required for the development and differentiation of multiple murine tissues (4, 9, 11, 12, 17). We hypothesize that deletion of SOX4, specifically in the prostate, will affect normal murine prostate development. Dr. Neal Copeland has provided us with mice that contain the endogenous SOX4 allele flanked by LOXP sites to facilitate CRE mediated deletion of SOX4. Here at Emory we already have a colony of mice containing the CRE transgene driven by the prostate specific Probasin promoter. Probasin is initially expressed at the onset of puberty (roughly two weeks of age) in all lobes of the prostate and seminal vesicles and mostly epithelial cells (19). We initially obtained SOX4^{fl/+} heterozygote mice and these mice are being bred to homozygosity as well as being crossed to the Probasin-CRE (Pb-CRE) mice to obtain homozygous SOX4 floxed males (SOX4^{fl/fl}) who are Pb-CRE positive. Currently we have obtained Pb-CRE negative, SOX4^{fl/fl} males and females as well as SOX4^{fl/+}, Pb-CRE positive males and females. Once male, prostate specific SOX4 knockout mice are obtained we will dissect out the prostate and harvest RNA and protein to assess SOX4 expression levels. This will provide a unique opportunity to investigate the expression status of direct SOX4 target genes predicted by our ChIP-chip analysis. Tissue sections will also be H&E stained for morphology analysis as well as immunohistochemical staining to determine the status of different prostate cell types.

Key Research Accomplishments:

- Expanded the known SOX4 target genes in the prostate to 282
- Identified 3,600 SOX4 binding sites in the proximal promoter of 3,470 different genes
- Developed a novel PBM k-mer based SOX4 binding site search algorithm in the perl programming language
- Identified biological pathways and processes SOX4 influences
- Significantly advanced the breeding of prostate specific SOX4 knockout mice

Reportable Outcomes:

- *Manuscripts:* The research presented in Aim 1 has been accepted for publication in *Cancer Research* and will be published on January 15, 2009 (13).
- *Abstracts:* The research in Aim 1 was presented as a poster at the 2008 Keystone meeting: Signaling Pathways in Cancer and Development.
- *Presentations:* All research presented in Aim I is presented as annually as an oral lecture as a requirement of my graduate program (Genetics and Molecular Biology).
- *Database:* All ChIP-chip and Expression profiling data has been deposited in the GEO database as required for publication under the Accession number: GEO11915
- *Funding Application:* All research presented in this report is part of an NIH Competitive Renewal application, applied for by my Principle Investigator Dr. Carlos Moreno.
- *Training:* As a student of the Genetics and Molecular Biology program I attend research seminars twice weekly and have taken 8 hours of course work comprising two classes: 1- a comprehensive Cancer Biology course, and 2- a introductory Bioinformatics course. My mentor and principle investigator, Dr. Carlos Moreno, has informally instructed me in the Perl Programming language as well as intensive direction in the analysis and data mining of microarray data from different platforms. In the next year I plan on writing and defending a dissertation consisting of the work presented in this report.

Conclusion:

In recent years various labs have utilized expression microarray data mining to identify a handful of SOX4 target genes. This report, for the first time, identifies the SOX4 target genes on a truly global scale. Interestingly, this data has highlighted a previously unknown function of SOX4. The vast array of transcription factor targets suggests SOX4 has a role in modulating other transcriptional programs towards a common goal. *In vivo* experiments presented in Aim 2 will aid our understanding of SOX4's role in prostate development and the consequences of prostate specific ablation of SOX4 will be studied and linked to our transcriptional target data.

One draw back from our ChIP-chip approach was that our NimbleGen chip only contained proximal promoter sequences. SOX4 has been reported to bind at least one enhancer in T-cells (18) and most likely affects other enhancers in our prostate model. Performing either ChIP-SEQ or ChIP-chip using a whole genome tiling array would lend more insight and truly define a global SOX4 regulatory network. Of particular interest to our lab is SOX4's role in WNT signaling. Our lab will explore the details of SOX4's interaction with β -catenin and how this affects the target genes SOX4 affects.

SOX4 has been shown to be overexpressed in prostate cancer as well as many other types of human cancers such as melanoma, medulloblastomas, glioblastomas and leukemias. Identifying the transcriptional programs SOX4 controls is a first step in elucidating how SOX4 promotes

carcinogenesis and evaluating SOX4 as a potential drug target in prostate cancer and other malignancies.

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Appendices:

I. Curriculum Vitae

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Education

Emory University, Atlanta, Georgia

- **Ph.D.** in Biomedical and Biological Sciences,
 - Program: Genetics and Molecular Biology - May, 2009
 - Dissertation: “Global Identification of Transcriptional Targets for *SOX4* in Prostate Cancer.”
 - Advisor: Dr. Carlos S. Moreno
 - GPA: 4.0

Emory University, Atlanta, Georgia

- **B.S.** in Biology – May, 2004
 - GPA: 3.4

Academic Awards and Fellowships

Department of Defense Predoctoral Training Grant in Prostate Cancer Research – 2006 – 2009

GDBBS Student Symposium, 2nd Place Poster Award - 2008

GDBBS Excellence in Teaching Award – 2007

NIH Predoctoral Training Grant – GMB, 2005 - 2006

Thomas Aliberti Scholar/Athlete Award – 2004

Research Experience

Doctoral Research:

Genetics and Molecular Biology, GDBBS, Emory University, Atlanta, Georgia, 2004-2009

(Advisor: Dr. Carlos Moreno - cmoreno@emory.edu)

- Analysis of the transcriptional targets for the oncogenic transcription factor *SOX4* using Chromatin Immunoprecipitation (ChIP), followed by DNA microarray and analysis with computational software developed by our lab.
- Investigation into the role of *SOX4* in prostate cancer formation using both a prostate specific over-expression and knockout mouse model.
- Improve treatment options for recurrent ovarian cancer by investigating whether an Aurora kinase family inhibitor can overcome Paclitaxel resistance in ovarian cancer cell lines.

Undergraduate Research:

Annual Report

Department of Neurology, Emory University School of Medicine, Atlanta, Georgia 2003-2004.

(Advisor: Dr. Enrique Torre – etorre@emory.edu)

- Investigation into localized transcription in neuronal axons grown both in culture and purified from mice.
- Analysis of the function of the chimeric, mutant gene *Wlds* and its role in slow Wallerian degeneration in neurons.

Teaching Experience**Teaching Assistant:**

Undergraduate Cancer Biology, Emory University, Spring 2006

(Professor: Dr. Gregg Orloff – gregg.orloff@emory.edu)

- Taught one lecture, assisted in student presentations, writing and grading tests, as well as tutoring students.

Undergraduate Tutoring:

- Served as a mentor and tutor for several undergraduates enrolled in Biology classes at Emory University – 100 hours

Additional Activities and Honors

- **Varsity Soccer**, Emory University – 2000-2003
 - Captain – 2003
 - UAA All-Conference Honorable Mention - 2002, 2003
 - Thomas-Aliberti Scholar/Athlete Award - 2004
- **Sigma Chi**, Beta Chi Chapter
- **USLlive Broadcaster** for the Atlanta Silverbacks – 2007-present

Peer Reviewed Publications

2009. **C.D. Scharer**, C.D. McCabe, M. Ali-Seyed, M.F. Berger, M.L. Bulyk, and C.S. Moreno. Genome-wide Location Analysis of the SOX4 Transcriptional Network in Prostate Cancer. *Cancer Research*, *in press*

2009. **C.D. Scharer**, N. Laycock, A.O. Osunkoya, S. Logani, J.F. McDonald, B.B. Benigno, and C.S. Moreno. Aurora kinase inhibitors synergize with paclitaxel to induce apoptosis in ovarian cancer cells. *Journal of Translational Medicine*, *in press*

2006. P. Liu, S. Ramachandran, M. Ali-Seyed, **C.D. Scharer**, N. Laycock, W. B. Dalton, H. Williams, S. Karanam, M. W. Datta, D. L. Jaye, and C. S. Moreno. SOX4 is a Transforming Oncogene in Human Prostate Cancer Cells. *Cancer Research*, **66**: 4011-4018.

Published Abstracts

C.D. Scharer and C.S. Moreno. Proteomics Analysis of Sox4 Reveals Post-Translational Modifications and Novel Protein-Protein Interactions [Abstract]. AACR Annual Meeting, April 18-22, 2009.

C.D. Scharer, C.D. McCabe, M.F. Berger, M.L. Bulyk, and C.S. Moreno. Whole Genome ChIP-chip Promoter Analysis Identifies Direct Transcriptional Targets for SOX4 in Prostate Cancer Cells [Abstract]. Signaling Pathways in Cancer and Development, Keystone Symposium, March 24-29, 2008.

Ali-Seyed, M, **C.D. Scharer**, and C.S. Moreno. SOX4 Participates in an Epidermal Growth Factor Receptor Positive Feedback Loop [Abstract]. Mechanisms & Models of Cancer, Cold Spring Harbor Laboratory, August 16-20, 2006.

II. Aim 1 Publication: Scharer et al. *Cancer Research*, in press

Q2 Genome-wide Promoter Analysis of the *SOX4* Transcriptional Network in Prostate Cancer Cells

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Abstract

SOX4 is a critical developmental transcription factor in vertebrates and is required for precise differentiation and proliferation in multiple tissues. In addition, *SOX4* is overexpressed in many human malignancies, but the exact role of *SOX4* in cancer progression is not well understood. Here, we have identified the direct transcriptional targets of *SOX4* using a combination of genome-wide localization chromatin immunoprecipitation–chip analysis and transient overexpression followed by expression profiling in a prostate cancer model cell line. We have also used protein-binding microarrays to derive a novel *SOX4*-specific position-weight matrix and determined that *SOX4* binding sites are enriched in *SOX4*-bound promoter regions. Direct transcriptional targets of *SOX4* include several key cellular regulators, such as *EGFR*, *HSP70*, *Tenascin C*, *Frizzled-5*, *Patched-1*, and *Delta-like 1*. We also show that *SOX4* targets 23 transcription factors, such as *MLL*, *FOXA1*, *ZNF281*, and *NKX3-1*. In addition, *SOX4* directly regulates expression of three components of the RNA-induced silencing complex, namely *Dicer*, *Argonaute 1*, and *RNA Helicase A*. These data provide new insights into how *SOX4* affects developmental signaling pathways and how these changes may influence cancer progression via regulation of gene networks involved in microRNA processing, transcriptional regulation, the *TGFβ*, *Wnt*, *Hedgehog*, and *Notch* pathways, growth factor signaling, and tumor metastasis. [Cancer Res 2009;69(2):OF1–9]

Introduction

The sex determining region Y-box 4 (*SOX4*) gene is a developmental transcription factor important for progenitor cell development and *Wnt* signaling (1, 2). *SOX4* is a 47-kDa protein that is encoded by a single exon and contains a conserved high-mobility group DNA-binding domain (DBD) related to the *TCF/LEF* family of transcription factors that mediate transcriptional responses to *Wnt* signals. *SOX4* directly interacts with β -catenin, but its precise role in the *Wnt* pathway is unknown (2). In adult mice, *SOX4* is expressed in the gonads, thymus, T-lymphocyte and pro-B-lymphocyte lineages, and to a lesser extent in the lungs,

lymph nodes, and heart (1). Embryonic knockout of *SOX4* is lethal around day E14 due to cardiac failure, and these mice also showed impaired lymphocyte development (3). Tissue-specific knockout of *SOX4* in the pancreas results in failure of normal development of pancreatic islets (4). *SOX4* heterozygous mice have impaired bone development (5), whereas prolonged expression of *SOX4* inhibits correct neuronal differentiation (6). These studies suggest a critical role for *SOX4* in cell fate decisions and differentiation.

Whereas *SOX2* is critical for maintenance of stem cells (7), *SOX4* may specify transit-amplifying progenitor cells that are the immediate daughters of adult stem cells and have been proposed to be the population that gives rise to cancer stem cells. In humans, *SOX4* is expressed in the developing breast and osteoblasts and up-regulated in response to progestins (8). *SOX4* is up-regulated at the mRNA and protein level in prostate cancer cell lines and patient samples, and this up-regulation is correlated with Gleason score or tumor grade (9). In addition, *SOX4* is overexpressed in many other types of human cancers, including leukemias, melanomas, glioblastomas, medulloblastomas (10), and cancers of the bladder (11) and lung (12). A meta-analysis examining the transcriptional profiles of human cancers found *SOX4* to be 1 of 64 genes up-regulated as a general cancer signature (12), suggesting that *SOX4* has a role in many malignancies. Furthermore, *SOX4* cooperates with *Evi1* in mouse models of myeloid leukemogenesis (13). Recently, we showed that *SOX4* can induce anchorage-independent growth in prostate cancer cells (9). Consistent with the concept that *SOX4* is an oncogene, three independent studies searching for oncogenes have found *SOX4* to be one of the most common retroviral integration sites, resulting in increased mRNA (14–16).

Despite these findings, the role that *SOX4* plays in carcinogenesis remains poorly defined. Whereas the transactivational properties of *SOX4* have been characterized (17), genuine transcriptional targets remain elusive. To date, three studies have used expression profiling of cells after either small interfering RNA (siRNA) knockdown or overexpression of *SOX4* to identify candidate downstream target genes (9, 11, 18). Very recently, 31 *SOX4* target genes were confirmed by chromatin immunoprecipitation (ChIP) in a hepatocellular carcinoma cell line (19). Although interesting, this study was limited by the fact that it focused on a specific tumor stage transition and did not use a genome-wide localization approach.

Here, we have performed a genome-wide localization analysis using a ChIP-chip approach to identify those genes that have *SOX4* bound at their proximal promoters in human prostate cancer cells. We have identified 282 genes that are high-confidence direct *SOX4* targets, including many genes involved in microRNA (miRNA) processing, transcriptional regulation, developmental pathways, growth factor signaling, and tumor metastasis. We have also used

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-08-3415

unique protein-binding DNA microarrays (PBM; refs. 20–22) to query the binding of recombinant *SOX4* to every possible 8-mer. The PBM-derived *SOX4* DNA binding data will further facilitate computational analyses of genomic *SOX4* binding sites. These data provide new insights into how *SOX4* affects key growth factor and developmental pathways and how these changes may influence cancer progression.

Materials and Methods

Cell culture and stable cell line construction. All cell lines were cultured, as described by American Type Culture Collection except LNCaP cells, which were cultured with T-Medium (Invitrogen). HA-tagged *SOX4* was cloned into the pHR-UBQ-IRES-eYFP-ΔU3 lentiviral vector (gift from Dr. Hihn Ly, Emory University), and stable cells were isolated, as previously described (23).

ChIP. Two 90% confluent P150s of both LNCaP-YFP and LNCaP-YFP/HA-*SOX4* or RWPE-1-YFP and RWPE-1-YFP/HA-*SOX4* cells were formaldehyde fixed and sonicated, and ChIP assay was performed, as described previously (23). Anti-HA 12CA5 or mouse IgG was used to immunoprecipitate protein-DNA complexes overnight at 4°C and collected using Dynal M280 sheep anti-mouse IgG beads for 2 h. Dynal beads were washed, protein-DNA complexes were eluted, and DNA was purified, as described previously (24). A detailed description of the ChIP-chip protocol can be found in Supplementary Methods. Anti-HA 12CA5, anti-Flag-M2 (Sigma-Aldrich), or mouse IgG was used to immunoprecipitate protein-DNA complexes overnight at 4°C. All PCR primers used in ChIP-PCR can be found in Supplementary Table S7.

ChIP-chip analysis. To determine the direct *SOX4* target genes on a global scale, we performed ChIP assays in triplicate from the LNCaP cell line stably expressing *SOX4* and in duplicate from a control cell line that expressed YFP alone. Immunoprecipitated and input DNA were subjected to whole genome amplification, Cy3/Cy5 fluorescent labeling, and

hybridization to the NimbleGen 25K human promoter array set. Input and immunoprecipitated DNA isolated from LNCaP-YFP and LNCaP-YFP/HA-*SOX4* cells were amplified using linker-mediated PCR as described previously (25). Amplified DNA was labeled and hybridized in triplicate by NimbleGen Systems, Inc., to their human 25K promoter array. This set consists of two microarrays that tile 4 kb of upstream promoter sequence and 750 bp of downstream intronic sequence on average, with a total genomic coverage of 110 Mb. Raw hybridization data were Z-score normalized, and ratios of immunoprecipitation to input DNA were determined for each sample. ChIPOTle software was used to determine enriched peaks using a 500-bp sliding window every 50 bp, as previously described (23). NimbleGen microarray data are available from the GEO database accession number GEO11915.

Luciferase assays. PCR fragments representing the binding sites in the *EGFR*, *ERBB2*, and *TLE1* genes were cloned in front of the pGL3-promoter luciferase construct (Promega). Primers sequences used can be found in Supplementary Table S7. LNCaP cells were transfected with 100 ng of TK-*Renilla* construct, 500 ng of pGL3-promoter vector alone and with cloned inserts, and 500 ng of either a *SOX4* or vector expression construct. Dual luciferase assays were performed 48 h posttransfection, according to the manufacturer's guidelines (Promega). All assays were performed in triplicate on separate days.

Quantitative real-time PCR. LNCaP cells were plated in six-well culture dishes and grown to 90% confluency before transfection with 1 μg of *SOX4* plasmid or vector control using Lipofectamine 2000 (Invitrogen). At 24 h posttransfection, total RNA was harvested using the RNeasy kit (Qiagen), and reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was performed using SYBR Green I (Invitrogen) on a Bio-Rad iCycler using 18s or β-actin as a control, and data were analyzed using the δCt method (26). All primers used in this study are listed in Supplementary Table S7.

Microarray analysis. Total RNA was isolated from three independent experiments of either vector control or *SOX4*-transfected LNCaP cells, as

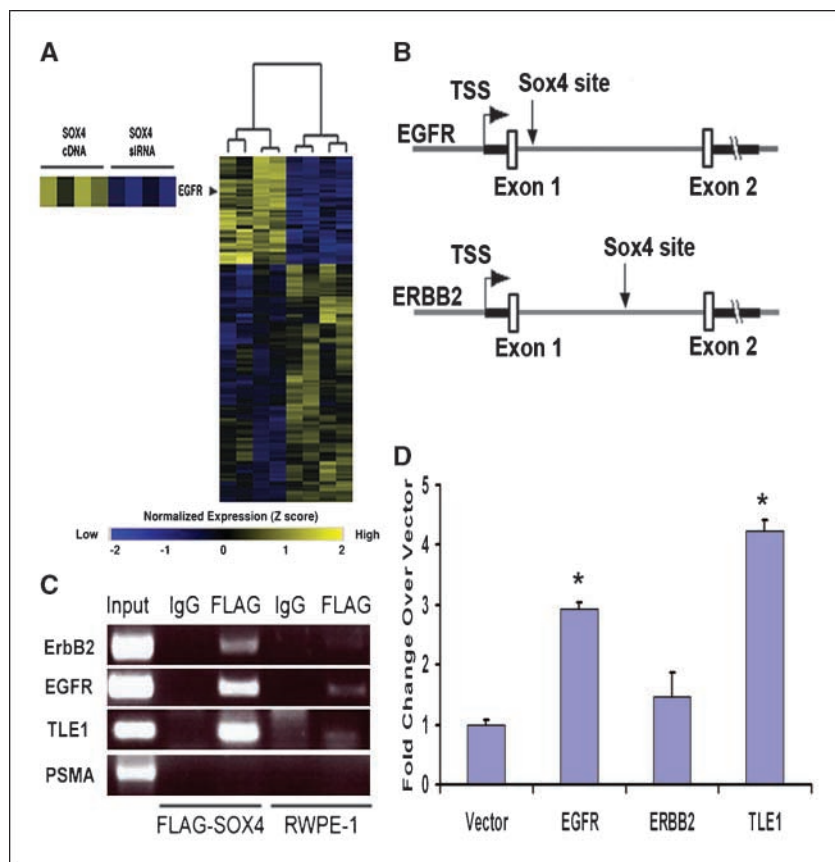
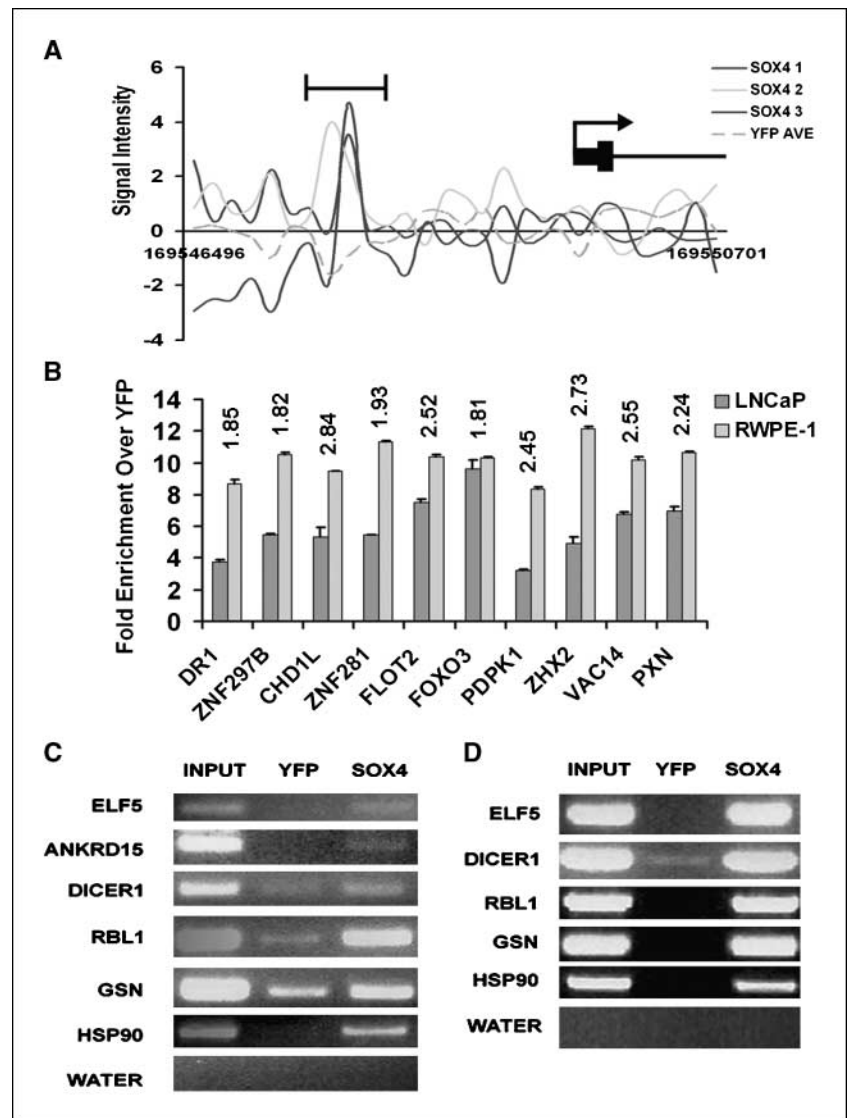


Figure 1. A, affymetrix U133A GeneChip microarray analysis of *SOX4* overexpression and knockdown in LNCaP prostate cancer cells. Overexpression of *SOX4* leads to increased *EGFR* expression, whereas siRNA knockdown of *SOX4* results in decreased *EGFR* expression. B, schematic showing the location of the *SOX4* binding site in the first intron of the *EGFR* (top) and *ERBB2* (bottom) genes. Arrows denote location of the *SOX4* binding site. C, ChIP assay of FLAG-*SOX4* bound to the introns of *EGFR*, *ERBB2*, and *TLE1*. *PSMA* is shown as a negative control. *SOX4* bound DNA is specifically amplified in the FLAG immunoprecipitation lane from FLAG-*SOX4* expressing cells (lane 3) and not control cells (lane 5) or with a nonspecific antibody (lanes 2 and 4). D, luciferase reporter assays with *SOX4* binding sites showing activation in the presence of *SOX4* compared with empty vector. *, $P < 0.01$ by Student's t test; bars, SD ($n = 3$ independent biological replicates performed on separate days).

Figure 2. A, graph showing enrichment in the three HA-SOX4 lanes over the average of the two YFP replicates for the *SOX4* target gene *FMO4*. Y axis is the signal intensity across the genomic coordinates on the X axis. B, qPCR ChIP analysis of 10 randomly selected genes verified in both the RWPE-1 and LNCaP cell lines. Graph shows fold enrichment of the HA-SOX4 immunoprecipitation over the YFP negative control immunoprecipitation. Numbers above the bars represent the mean log₂ of fold enrichment of ChIP-chip signal for the probes contained in the peak relative to YFP. Bars, SD (*n* = 3 technical replicates). C and D, genes that were verified by conventional ChIP assay. HA-SOX4 and YFP cells were subjected to conventional ChIP followed by PCR in both the LNCaP (C) and RWPE-1 (D) prostate cell lines. Six genes verified in the LNCaP cell lines and five in the RWPE-1 cell lines.



described above. Each transfection was performed in triplicate, and each sample was hybridized in duplicate, creating six data points for each condition. Total RNA was submitted to the Winship Cancer Institute DNA Microarray Core facility.⁸ All samples showed RNA integrity of 8.3 or greater using an Agilent 2100 Bioanalyzer. RNA was hybridized to the Illumina Human6 v2 Expression Beadchip that query roughly 47,000 transcripts with 48,701 probes, and after normalization, significantly changed probes were calculated using significance analysis of microarrays (SAM) software (27). Settings for SAM were two-class unpaired ($\times 4$ versus vector control) imputation engine (10 nearest neighbor), permutations (500), RNG seed (1234567), Delta (1.316), fold change (1.5), and false discovery rate (0.749%). Microarray data are available in the GEO database accession number GEO11915.

Immunoblotting. Cells were lysed in lysis buffer [0.137 mol/L NaCl, 0.02 mol/L TRIS (pH 8.0), 10% glycerol, and 1% NP40], and 50 μ g total lysate were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose for immunoblotting. Immunoblots were probed with polyclonal rabbit *SOX4* antisera described previously (9) and *DICER* (Santa Cruz). To control for

equal loading, immunoblots were also probed with a mouse monoclonal antibody to protein phosphatase 2A (*PP2A*) catalytic subunit (BD Biosciences).

Results

SOX4 transcriptionally activates *EGFR*. Using expression profiling to determine the genes whose mRNA levels change when *SOX4* is either overexpressed or eliminated using siRNA (9), we identified *EGFR* as a candidate *SOX4* transcriptional target (Fig. 1A). Analysis of the promoter and first intron of *EGFR* and other family members with CONFAC software (28) revealed the presence of potential *SOX4* binding sites within the first intron of *EGFR* and *ERBB2* (Fig. 1B). CONFAC functions by identifying the conserved sequences in the 3-kb proximal promoter region and first intron of human-mouse orthologue gene pairs and then identifying transcription factor binding sites (TFBS), defined by position weight matrices from the MATCH software (29), which are conserved between the two species (28).

Whereas limited commercial antibodies exist for *SOX4* and show activity in immunoblots, in our hands, none of them have been

⁸ <http://microarray.cancer.emory.edu/>

useful in a ChIP assay. Therefore, we used epitope-tagged *SOX4*, as described in other *SOX4* ChIP studies (9, 19). Although the FLAG epitope tag was not tested directly for activity, a glutathione *S*-transferase (GST)-*SOX4* construct showed binding to a known *SOX4* motif and not a control motif (Supplementary Fig. S2B), validating that the epitope tag does not interfere with *SOX4* binding. To determine if *SOX4* directly bound the *EGFR* and *ERBB2* enhancers, we performed ChIP analysis on RWPE-1 prostate cancer cells stably infected with FLAG-*SOX4* or a control lentiviral vector. DNA representing the predicted *SOX4* sites was specifically amplified from the FLAG-*SOX4* cell line and not from the control cell line, indicating that *SOX4* binds to intronic sequence of *EGFR* and *ERBB2* (Fig. 1C). *EGFR* is expressed in RWPE-1 cells, but not in LNCaP cells, and *SOX4* did not bind to these sequences in LNCaP cells (data not shown).

To characterize the transcriptional effect of *SOX4* levels on the regions bound by *SOX4* in ChIP assays, the amplified ChIP fragments were cloned in front of a minimal promoter luciferase reporter plasmid and tested in transient transfections in LNCaP cells. Compared with a vector control, *SOX4* significantly increased transcription of the *EGFR* fragment 3-fold and the *TLE1*-positive control fragment roughly 4-fold. Although not found significant, *ERBB2* was activated 1.5-fold compared with the vector control (Fig. 1D). Consistent with microarray data, *SOX4* transcriptionally activates the *EGFR* enhancer.

Genome-wide localization analysis. To determine the direct *SOX4* target genes on a global scale, we performed ChIP assays in triplicate from the LNCaP HA-*SOX4* stable cell line and in duplicate from the control LNCaP-YFP cell line. Peaks ($P < 0.001$) that overlapped in at least two of the three data sets and were not

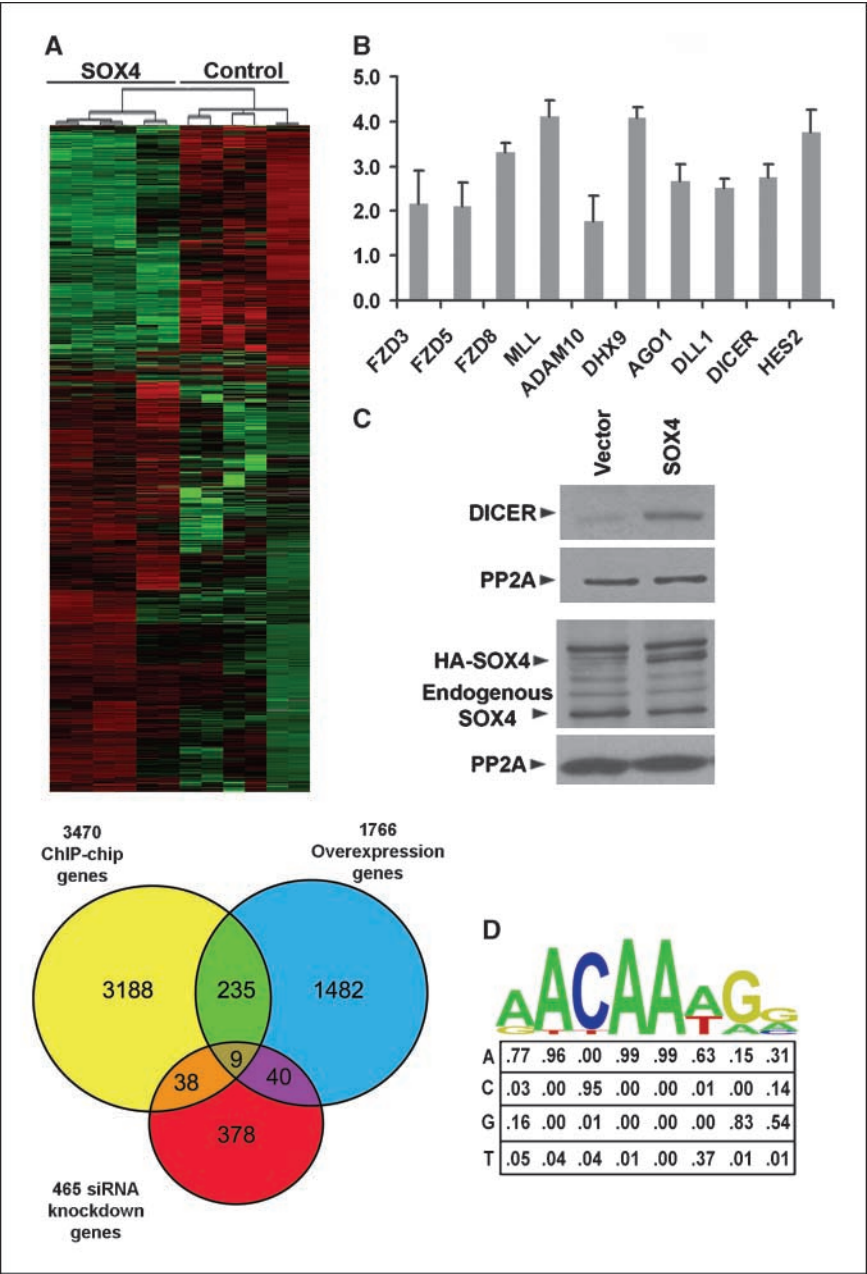


Figure 3. A, heat map (top) illustrating Illumina expression data of the 1,766 significant genes, as determined by SAM analysis. Red, overexpressed genes; green, underexpressed genes. Venn diagram (bottom) depicts the overlap between 3,470 ChIP-chip *SOX4* direct target genes, the Illumina expression data set of 1,766 genes, and the Affymetrix expression data set of 465 genes. B, qPCR expression analysis of *SOX4* direct target genes after *SOX4* overexpression in LNCaP cells. All 10 genes were up-regulated over a vector control transfection, similar to values determined by the Illumina array with a P value of <0.005 by Student's t test. Bars, SD ($n = 3$ independent biological replicates performed on separate days). C, *DICER* protein expression is up-regulated by *SOX4*. HA-*SOX4* or vector control was transfected into LNCaP cells, and immunoblots were probed for *DICER*, *SOX4*, and PP2Ac as a loading control. D, PBM-derived 8-mer PWM for *SOX4* displayed both graphically and numerically for each base position derived from incubation of recombinant GST-*SOX4*-DBD with a universal "all 8-mer" double-stranded DNA protein-binding microarray. With stringent criteria (core similarity, >0.85 ; matrix similarity, >0.75) we find 60% of the peaks in the 282 high-confidence promoters contain *SOX4* binding sites.

F2 present in the LNCaP-YFP cell line were called significant (Fig. 2A). Based on these variables, we classified 3,600 significant, overlapping peaks as *SOX4* target sequences. Because some transcription start sites (TSS) are quite close to each other (<3 kb), it was not always possible to assign a unique gene to every peak. In addition, many genes had multiple peaks in their promoters, and thus, we mapped the 3,600 peaks to 3,470 different genes (Supplementary Table S1).

ST1 To verify the set of 3,600 *SOX4* peaks, 28 candidate *SOX4* target sites representing a range of *P* values in promoters of genes of biological interest were chosen, primers were designed around the peaks and enrichment was verified by conventional ChIP. Ten of these 28 candidates were analyzed by ChIP qPCR and 18 by ChIP-PCR. Overall, 24 of 28 (86%) of the candidate targets were confirmed, validating our data set. All 10 of the peaks chosen to validate by qPCR were reproducibly enriched over the *YFP* control in both the LNCaP-HA-SOX4 cell line and the RWPE-1 cell line (Fig. 2B). Of the target sites validated by conventional PCR, 14 of 18 genes were confirmed in both the LNCaP and RWPE-1 cell lines, whereas a mock, control PCR was negative (Fig. 2C and D; data not shown). The only exception was *ANKRD15*, which was enriched only in the LNCaP cell line and not in the RWPE-1 line.

F3 Target gene expression analysis. To determine whether *SOX4* binding affects transcription of the 3,470 genes that have *SOX4* bound at their promoters, we performed whole genome expression analysis on LNCaP cells after transfection with *SOX4* or a control vector. To increase the likelihood of identifying direct *SOX4* targets, total RNA was isolated at a relatively early time point (24 hours posttransfection) and hybridized to Illumina Human 6-v2 whole genome arrays. A total of 1,766 genes were changed at least 1.5-fold with a false discovery rate of 0.749% (Fig. 3A; Supplementary Table S2). Of those 1,766 genes, 244 were also direct *SOX4* targets by ChIP-chip analysis (Fig. 3A; Supplementary Table S3). Seven of these genes were confirmed by qPCR (Fig. 3B).

ST2 Our previous expression profiling of LNCaP cells after *SOX4* siRNA knockdown (9) identified 465 downstream targets, and we confirmed that *SOX4* regulates the expression of *DICER*, *DLL1*, and *HES2* in LNCaP cells by qPCR (Fig. 3B). We further confirmed *SOX4* regulation of *DICER* at the protein level (Fig. 3C). Out of those 465 candidate targets, 47 genes overlapped with the 3,470 ChIP-chip targets, increasing the number of direct *SOX4* targets to 282 genes (Fig. 3A; Supplementary Table S3). We classified these 282 genes bound by *SOX4* in ChIP-chip and significantly changed by expression profiling as high confidence direct *SOX4* target genes. Nine genes (*PIK4CA*, *DHX9*, *BTN3A3*, *CDK2*, *MVK*, *ADAM10*, *RYK*, *ISG20*, and *DBI*) overlapped in all three data sets. The transcription factor *SON* and purine biosynthetic enzyme *GART*, two genes on chromosome 21 that are transcribed in opposite directions and regulated by a bidirectional promoter, were affected in opposite ways. *SON* was activated by *SOX4* 1.8-fold, as detected by *SOX4* overexpression, whereas *GART* was increased almost 3-fold as determined by *SOX4* siRNA knockdown, suggesting that *SOX4* regulates the directionality of this promoter.

ST3 We next analyzed the *P* values of the peaks in our ChIP-chip data set, comparing the *P* values of the genes that were altered by transient overexpression of *SOX4* with those that were not (Supplementary Fig. S2). We found no difference in the distributions of the ChIP-chip *P* values for those genes that were changed in expression profiling experiments and those that were not. Thus, based on our ChIP-chip validation experiments and the similar *P*-value distributions, we conclude that *SOX4* is genuinely bound at

the promoters of the 3,188 genes that did not change but that *SOX4* by itself is not limiting or sufficient to generate changes in transcription without corresponding changes in the cellular context, such as activation of cofactors or signaling pathways.

Novel *SOX4* position weight matrix. To facilitate computational analyses of *SOX4* DNA binding sites, we sought to determine the DNA binding preferences of *SOX4* using universal PBMs (20). This universal PBM array allows recombinant *SOX4* protein to interact with and bind every possible 8-mer, thus allowing *in vitro* binding site specificities to be calculated.

We generated an NH₂ terminal, GST-SOX4-DBD fusion protein, expressed and purified it from *E. coli*, and tested for activity (Supplementary Fig. S3). The GST-SOX4-DBD was incubated with the protein binding microarray and a novel position weight matrix (PWM; RWYAAWRV) was calculated from the PBM data (Supplementary Table S4) using the Seed-and-Wobble algorithm (Fig. 3D; ref. 20). Three groups have previously reported similar binding site sequences for *SOX4*: AACAAAG (30), AACAAT (31), and WWCAAWG (19). Our PWM confirms the *SOX4* core binding sequence of the previously known binding sites but there are some differences in the specificity at the 1st and 7th positions and we find a bias toward A, C, and G at the 8th position. These differences could be due to the fact that earlier reports used no more than 31 sequences to develop the binding motif, whereas our study queried every possible 8-mer.

SOX4 peaks contain SOX4 binding sites. Using our newly derived PWM, we applied CONFAC software (28) to analyze the enriched sequences for the presence of *SOX4* binding sites. We analyzed the sequences of the peaks in the promoters of our 282 high confidence genes against 10 sets of control promoter sequences to see if *SOX4* sites were enriched in our target gene set. Control promoter peaks of equal size to *SOX4* peaks were chosen randomly from sequences covered by the NimbleGen array, and each control set contained equal total sequence coverage as our 282 high confidence peaks. With stringent criteria (core similarity, >0.85; matrix similarity, >0.75), we find that 60% of the peaks contain *SOX4* binding sites. *SOX4* sites were significantly enriched relative to 10 sets of random promoter sequence by Mann-Whitney *U* test using Benjamini correction for multiple hypothesis testing (*q* < 0.0019).

To further characterize the *SOX4* binding sites, we searched the entire set of 3,600 *SOX4* peaks and 10 equal sets of random promoter sequence for the presence of PBM-bound k-mers (here, ungapped 8-mers). The specificity of PBM k-mers can be quantified by the enrichment score (ES), which ranges from -0.5 to 0.5 (32). We analyzed the enrichment of PBM k-mers with 0.45 > ES > 0.40 (moderate) and ES > 0.45 (stringent). Whereas both *SOX4*-bound peaks and random promoter sequence contained moderate and stringent k-mers, *SOX4* peaks contained significantly more stringent (*P* = 0.0002) and moderate (*P* = 1.08×10^{-5}) k-mers by two-tailed Mann-Whitney test (Supplementary Fig. S4).

To investigate interaction with protein partners that may increase *SOX4* affinity for poor matching sites *in vivo*, we searched for enrichment of cooccurring TFBS in the *SOX4* peaks. We applied CONFAC software to search the sequences for the presence of co-occurring transcription factor binding sites within the same peak (Table 1). Using the same criteria as above, we determined that the *E2F* family had the most frequently co-occurring motif (similar to TTTCGCGC, *q* = 1.78×10^{-11}). Interestingly, ingenuity pathway analysis (IPA) identified cell cycle as a functionally enriched process in the 3,470 *SOX4* target genes (*P* = 0.00916), suggesting

Table 1. Benjamini corrected *q* values for co-occurring transcription factor binding sites

Transcription factor	Family	Benjamini corrected <i>q</i> value
E2F4	E2F	1.78E-11
E2F1	E2F	3.06E-11
PAX5	Paired box	2.07E-10
WHN	Forkhead	2.94E-10
SMAD3	SMAD	1.82E-09
SMAD4	SMAD	3.33E-09
MYC	MYC	6.25E-09
NFKAPPAB	NF- κ B	2.95E-08
LEF1/TCF1	LEF	1.12E-06

that part of *SOX4*'s function is to control the expression of genes involved in cell cycle progression.

CONFAC analysis identified other significant TFBS motifs enriched in the *SOX4* peaks (Table 1), including those for transcription factors in the *TGF β* , *Wnt*, and *NF- κ B* pathways. *SOX4* modulates *Wnt* signaling via interaction with β -catenin and the *TCF4* transcription factor (2), suggesting a possible role for *SOX4* in transcriptionally modulating *Wnt* signals. We confirmed the recent report that *SOX4* cooperates with constitutively active β -catenin to activate TOP-Flash luciferase reporters (2) and found that *SOX4* synergistically induces activation of these constructs, further highlighting a role for *SOX4* in the *Wnt* pathway (Supplementary Fig. S5).

***SOX4* target genes.** To determine the biological processes and functions of the *SOX4* targets, we performed a gene ontology analysis using DAVID software (33) on the 282 high confidence *SOX4* targets. Among the *SOX4* targets were 23 transcription factors (Table 2), and DAVID analysis determined that the top annotations were transcription ($P = 3.7 \times 10^{-18}$), transmembrane ($P = 5.59 \times 10^{-10}$), and protein phosphorylation/dephosphorylation ($P = 3.5 \times 10^{-18}/6.6 \times 10^{-7}$). These findings are paralleled by expression profiling of *SOX4* overexpression in HU609 bladder carcinoma cells where top annotated functions were signal transduction and protein phosphorylation (11).

Commercial IPA software⁹ identified biological pathways and functions that are enriched in our 282 high confidence targets, 1,766 significant genes identified by SAM analysis, and the 3,470 unique genes that had *SOX4* bound at their promoters in ChIP-chip. As anticipated, among the most significant annotations were cell cycle, cancer, and tissue development. In the significant expression data set of 1,766 genes, we observed an up-regulation of three Frizzled family receptors, *FZD3*, *FZD5*, and *FZD8*, as well as the downstream transcription factor *TCF3*. Overall, IPA analyses discovered key components of the *EGFR*, *Notch*, *AKT-PI3K*, miRNA, and *Wnt- β -catenin* pathways as *SOX4* regulatory targets. Based on these findings, we built *SOX4* regulatory networks found in prostate cancer cells (Fig. 4 and Supplementary Fig. S6). *SOX4* target genes comprise key pathway components, such as ligands (*DLL1* and *NGR1*), receptors (*FZD5* and *PTCH1*), an AKT regulatory kinase (*PDPK1*), and downstream transcription factors (*FOXO3* and *HES2*). In addition, *SOX4* activates expression of

tenascin C, an extracellular matrix protein that is a target of *TGF β* signaling (34) and β -catenin (35). In addition, *SOX4* regulates three components of the RNA-induced silencing complex (RISC) complex, *DICER*, *Argonaute 1* (*AGO1*), and *RHA/DHX9* (Supplementary Table S3). We confirmed these data by qPCR (Fig. 3B) and Western blot for *DICER* (Fig. 3C).

Gene set enrichment analysis (GSEA; ref. 36) and GSEA leading edge analysis (37) of these gene sets identified *TGF β* -induced *SMAD3* direct target genes (Supplementary Table S5) as enriched in *SOX4* target genes. *SOX4* is up-regulated by *TGF β -1* treatment (4, 38), and we found *SMAD4* sites are significantly enriched in the *SOX4* ChIP-chip peaks (Table 1), suggesting that *SOX4* affects key developmental and growth factor signaling pathways in prostate cancer cells at both the transmembrane signaling and transcriptional levels.

Discussion

Whereas many studies have identified *SOX4* as a crucial developmental transcription factor that is often overexpressed in many types of malignancies, little is known of what *SOX4* regulates in cancer cells. We have used a ChIP-chip approach to report the first genome-wide localization analysis of *SOX4* and mapped 3,600 binding peaks that represent 3,470 unique genes possibly under the transcriptional control of *SOX4*. We have also identified 1,766 genes that respond to increased *SOX4* levels by whole genome expression profiling. Integration of these data sets mapped 282 high-confidence direct targets in the *SOX4* transcriptional network. In addition, we have used protein-binding microarrays

Table 2. DAVID analysis identified 23 transcription factors present in our high confidence *SOX4* target genes

Entrez ID	Symbol	Microarray fold change
196528	ARID2	1.99
2001	ELF5	-2.65
3169	FOXA1	-2.47
2976	GTF3C2	-3.12
64412	GZF1	2.42
84458	LCOR	2.41
4173	MCM4	1.55
58508	MLL3	2.06
10933	MORF4L1	2.07
8031	NCOA4	2.64
4784	NFIX	-2.83
4824	NKX3-1	-4.53
7799	PRDM2	2.48
5933	RBL1	1.80
55509	SNFT	-2.32
6722	SRF	-2.03
54816	SUHW4	-1.93
9412	SURB7	-2.24
9338	TCEAL1	-1.57
7718	ZNF165	1.53
7738	ZNF184	1.66
23528	ZNF281	1.71
30834	ZNRD1	-1.63

NOTE: Gene ontology term: transcription, DNA dependent ($P = 3.7 \times 10^{-18}$).

⁹ <http://www.ingenuity.com>

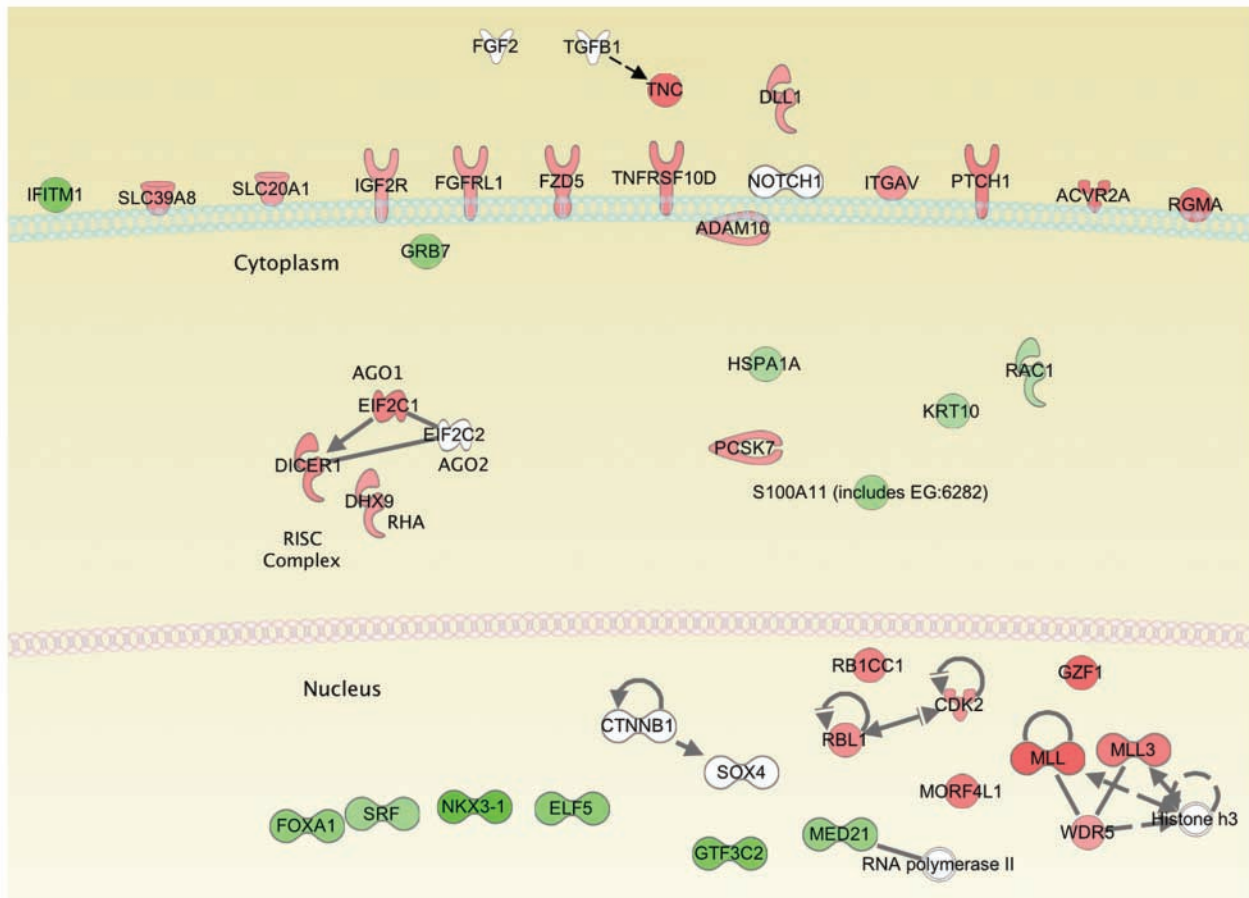


Figure 4. IPA analysis of direct target genes graphically illustrating the cellular location of the *SOX4* transcriptional target genes. *SOX4* regulates a host of nuclear and membrane localized proteins, as well as multiple components of the RISC complex. Red, target genes up-regulated by *SOX4*; green, down-regulated genes; white, genes for which no expression change was detected.

to determine a novel PWM specific for *SOX4* and show that our ChIP-chip predicted peaks are significantly enriched for *SOX4* binding sites. These data provide several new insights into the roles that *SOX4* plays in the cell.

***SOX4* direct target genes.** Although only 10% of the significant differentially expressed genes overlapped with the ChIP-chip data, this is likely a conservative estimate because the NimbleGen 25K promoter array only queries proximal promoter sequences and not more than 1 kb downstream of the TSS. We found that *SOX4* binds *EGFR* and *ERBB2* in the first intron over 20 kb downstream of the TSS (Fig. 1D), and unsurprisingly, we did not detect *EGFR* or *ERBB2* in our ChIP-chip experiment. Thus, more of the 1,900 genes that responded to changes in *SOX4* mRNA levels (but were not detected by ChIP-chip) could still be direct targets. Excellent candidates would be the 40 genes that responded to *SOX4* on both microarray platforms, such as the *IL6* receptor, *SOX12*, and *NME1* (Supplementary Table S6). Whereas 3,600 is a fairly large number of *SOX4* bound regions, some background can be expected. Nevertheless, we were able to validate 24 of 28 (86%) candidate binding sites chosen, adding confidence to our data set. In fact, an even higher number of over 4,200 genomic binding sites had been previously observed for *c-Myc* in ChIP-positron emission tomography whole genome studies (39). Whole genome tiling arrays or

ChIP-seq could provide additional binding sites that may show more overlap with the Illumina expression data set.

Conversely, many of the bound genes may not respond to changes in *SOX4* mRNA levels alone but to multiprotein activator complexes of which *SOX4* is only one component. Furthermore, the stability of *SOX4* bound to a promoter could be greater than unbound *SOX4*, limiting the effects observed by siRNA knockdown. In different cell types or cellular contexts, *SOX4* may activate a different subset of these genes. Of the 31 *SOX4* target genes reported by Liao and colleagues (19), only six are represented in our NimbleGen data set and three found to be changed in our Illumina expression profiling data set. The small overlap could be due to the fact that those genes were identified in hepatocellular carcinomas, whereas we have examined prostate cancer cells. Interestingly, *DKK1* was one of the six genes that overlapped in both data sets, further implicating *SOX4* in the *Wnt* pathway. Because *SOX4* is known to interact with β -catenin and other coactivators, it may be poised at many of these promoters to enable responses to developmental signals from the *Wnt* or *TGF β* pathway.

Receptor and signaling regulation. Our data suggest that *SOX4* regulates cellular differentiation through a variety of transcription factors and receptors. *SOX4* is up-regulated in response to numerous external ligands ranging from *TGF β* (38)

and *BMP-6* (40) to parathyroid hormone and progesterone (8). Previous work has shown that *SOX4* directly signals from *IL-5R α* (41), and here, we have shown that *SOX4* directly regulates *EGFR* (Fig. 1). Membrane receptors in the *SOX4* transcriptional network also include Frizzled family members *FZD3*, *FZD5*, *FZD8*; the Hedgehog receptor *PTCH-1*; the Notch ligand *DLL1*; TRAIL decoy receptor *TNFRSF10D*; and other growth factor receptors, such as *FGFRL1* and *IGF2R*. DAVID analysis also revealed protein phosphorylation/dephosphorylation ($P = 3.5 \times 10^{-18}/6.6 \times 10^{-7}$) and transcription ($P = 3.7 \times 10^{-18}$) are enriched annotations, identifying 23 transcription factors that are direct targets of *SOX4*. This evidence suggests that *SOX4* regulates signaling events both at the external input level and the internal output or transcription level. This regulation could be direct, as with *IL-5R α* , or through the transcriptional targets *SOX4* activates.

Transcription factors and *SOX4*. Here, we have reported DNA binding specificity data for *SOX4*, which will improve computational analyses for *SOX4* specific binding sites. Our data confirm the known SOX family core-binding motif and add new specificity at the 1st, 7th, and 8th positions. Whereas crystal structure evidence from *SOX2* has shown the importance of the core-binding motif, it is possible that the specificity for *SOX4* is enhanced outside of the core motif at the extra positions. A limitation of these data is that we did not assess how other DNA binding proteins influence the sequences to which *SOX4* can bind. The enrichment of *SMAD4* sites is particularly interesting in light of the GSEA results, which suggest that *SOX4* regulates many *TGF β* target genes, including *Tenascin C*. Thus, we hypothesize that *SOX4* may physically interact with *SMAD4* in response to *TGF β* signals. Experiments to test this hypothesis are under way. Nevertheless, evidence points to a role for *SOX4* in modulating other transcriptional programs via hierarchical regulation of 23 downstream transcription factors.

***SOX4* and cancer.** Based on the target genes we identified, *SOX4* seems to influence cancer progression in several ways. First, it plays a key role in the activation of and response to developmental pathways, such as *Wnt*, *Notch*, *Hedgehog*, and *TGF β* . Second, *SOX4* inhibits differentiation via repression of transcription factors, such as *NKX3.1*, and activation of *MLL* and *MLL3*, two histone H3 K4 methyltransferases that induce activation of *HOX* gene expression (42). *MLL* methyltransferase complexes also facilitate *E2F* activation of S-phase promoters, facilitating cell cycle progression. Activation of *MLL* also suggests a mechanism for the role of *SOX4* in myeloid leukemogenesis, because *MLL* is a critical oncogene that is often translocated or amplified in this disease (43). Thirdly, *SOX4* targets growth factor receptors, such as *EGFR*, *FGFRL1*, and *IGF2R*, enhancing proliferative signals in tumors and potentially activating the *PI3K-AKT* pathway. Mice heterozygous for *NKX3.1* and *PTEN* in the prostate develop prostate adenocarcinomas and metastases to the lymph node (44). Thus, our data suggest that *SOX4* may promote prostate cancer progression directly through *NKX3.1* repression and indirectly through *PI3K-AKT* activation. Finally, *SOX4* seems to promote metastasis via up-regulation of *tenascin C*. Recently, both *SOX4* and *tenascin C* were shown to enhance metastasis of breast

cancer cells to the lung (45), as has the *TGF β* pathway, which activates their expression (46). Other metastasis-associated *SOX4* target genes include *integrin α_V* and *Rac1*. *Rac1* was recently shown to control nuclear localization of β -catenin in response to *Wnt* signals (47).

***SOX4* regulates components of the RISC complex and small RNA pathway.** miRNAs are small noncoding RNA species that regulate the translation and stability of mRNA messages for hundreds of downstream target genes via partial complementarity to short sequences in the 3' untranslated regions of mRNAs. The RISC, which is composed of *AGO1* or *AGO2*, *TRBP*, and *Dicer* processes miRNAs from precursors (pre-miRNA) to their mature form, cleaves target mRNAs, and participates in translational inhibition. *RNA Helicase A (RHA/DHX9)* interacts with the RISC complex and participates in loading of small RNAs into the RISC complex (48). We observed that three components of the RISC complex, *DICER*, *AGO1*, and *RHA/DHX9*, are high-confidence direct targets of *SOX4* (Supplementary Table S3), and we confirmed these data by qPCR (Fig. 3B). *Dicer* has been independently observed to be overexpressed in prostate cancers (49).

In addition, we observed that *Toll-like receptor 3 (TLR3)*, which binds to double-stranded RNAs, induces gene silencing, and can induce apoptosis (50), was induced 2.8-fold upon overexpression of *SOX4*. This induction may be indirect because *TLR3* was not detected by ChIP-chip, but we cannot exclude the possibility that *SOX4* may directly regulate *TLR3* from a distal or intronic enhancer.

Our observation that *SOX4* targets three genes important in small RNA processing is of particular interest in light of the role of *SOX4* in development and cancer progression. miRNAs have been implicated in numerous physiologic processes from development to oncogenesis. miRNAs can also act as suppressors of breast cancer metastasis via targeting of *tenascin C* and *SOX4* (45) and as promoters of breast cancer metastasis (51). The finding that *SOX4* can affect expression of multiple components of the RISC complex also provides insight into why long-term loss of *SOX4* induces widespread apoptosis (9, 18). In summary, these data shed light on the mechanisms and pathways through which *SOX4* may exert its effects during development and cancer progression. Further studies are necessary to elucidate the precise role of *SOX4* in the functioning of these pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/3/2008; revised 10/8/2008; accepted 11/3/2008.

Grant support: National Cancer Institute R01 CA106826 (C.S. Moreno), NIH/NHGRI grant R01 HG003985 (M.F. Berger and M.L. Bulyk), DOD CDMRP Prostate Cancer Predoctoral Training Fellowship PC060145 (C.D. Scherer), and Postdoctoral Training Fellowship PC060114 (C.D. McCabe).

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We thank Dr. Maja Ordanic-Kodani at Winship Cancer Institute Microarray Core Facility for performing Illumina microarray labeling and hybridization, Robert Karaffa at the Emory University FlowCore for cell sorting, Dr. Hinh Ly for IRES-eYFP lentiviral vector, and Dr. Anita Corbett for pGEX-4T-1 plasmid.

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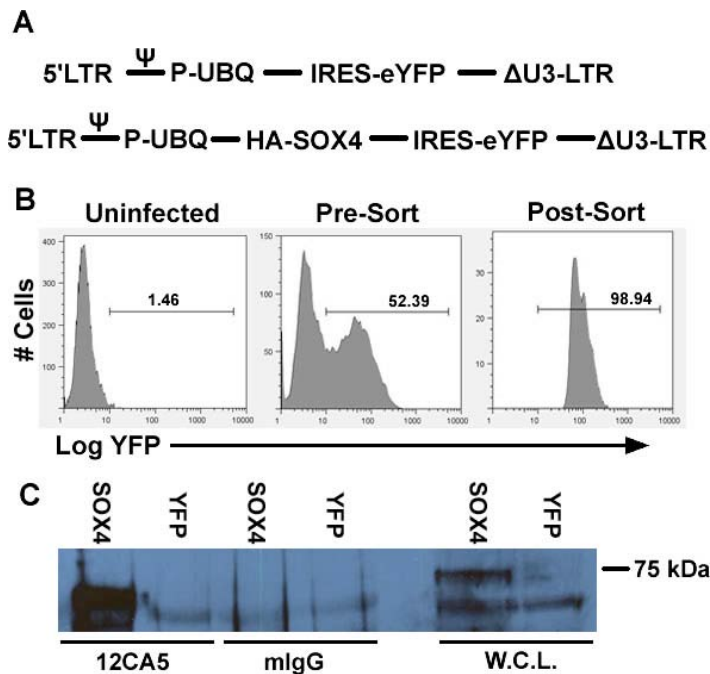


Figure 1: (A) Schematic diagram of the lentiviral constructs used to stably infect LNCaP and RWPE-1 prostate cancer cells showing the locations of LTRs and promoters. The top figure represents the control, eYFP only construct, and the lower figure represents the HA-SOX4 construct. (B) Histogram charts showing the control uninfected, pre-sorted and post-sorted cell populations. Lower axis displays YFP signal intensity. (C) Immunoblot showing that HA-SOX4 is expressed and specifically immunoprecipitated from the LNCaP-HA-SOX4 cell line and not the control LNCaP-YFP cell line.

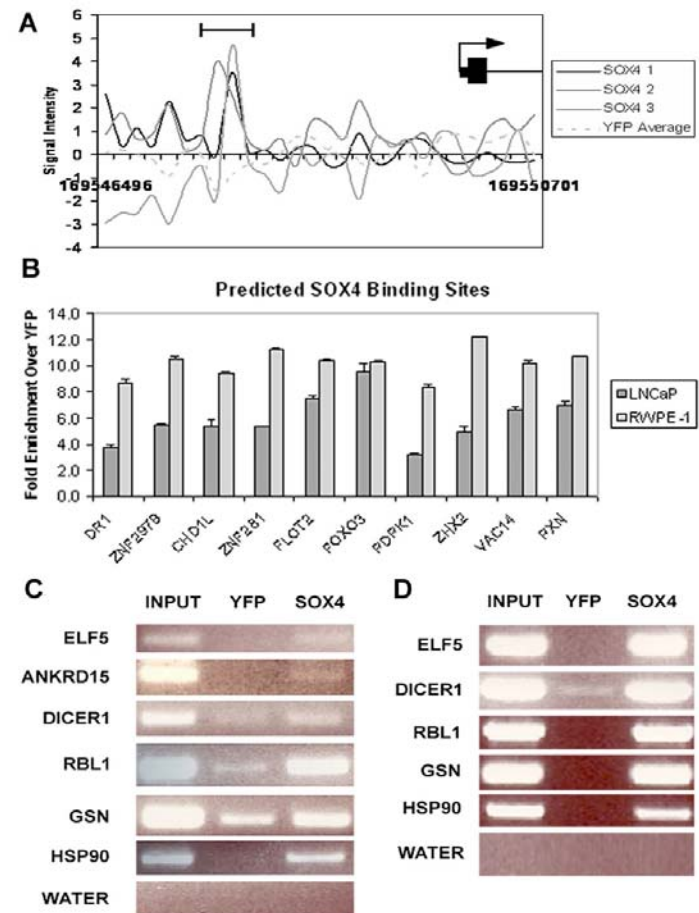


Figure 2: (A) Graph showing enrichment in the three HA-SOX4 lanes over the average of the two YFP replicates for the gene FMO4. (B) QRT-PCR analysis of 10 randomly selected genes verified in both the RWPE-1 and LNCaP cell lines. Graph shows fold enrichment of the HA-SOX4 IP over the YFP control IP. (C) Genes that were verified by conventional ChIP assay. LNCaP-HA-SOX4 and LNCaP-YFP cells were subjected to conventional ChIP followed by PCR in both the LNCaP and RWPE-1 prostate cell lines.

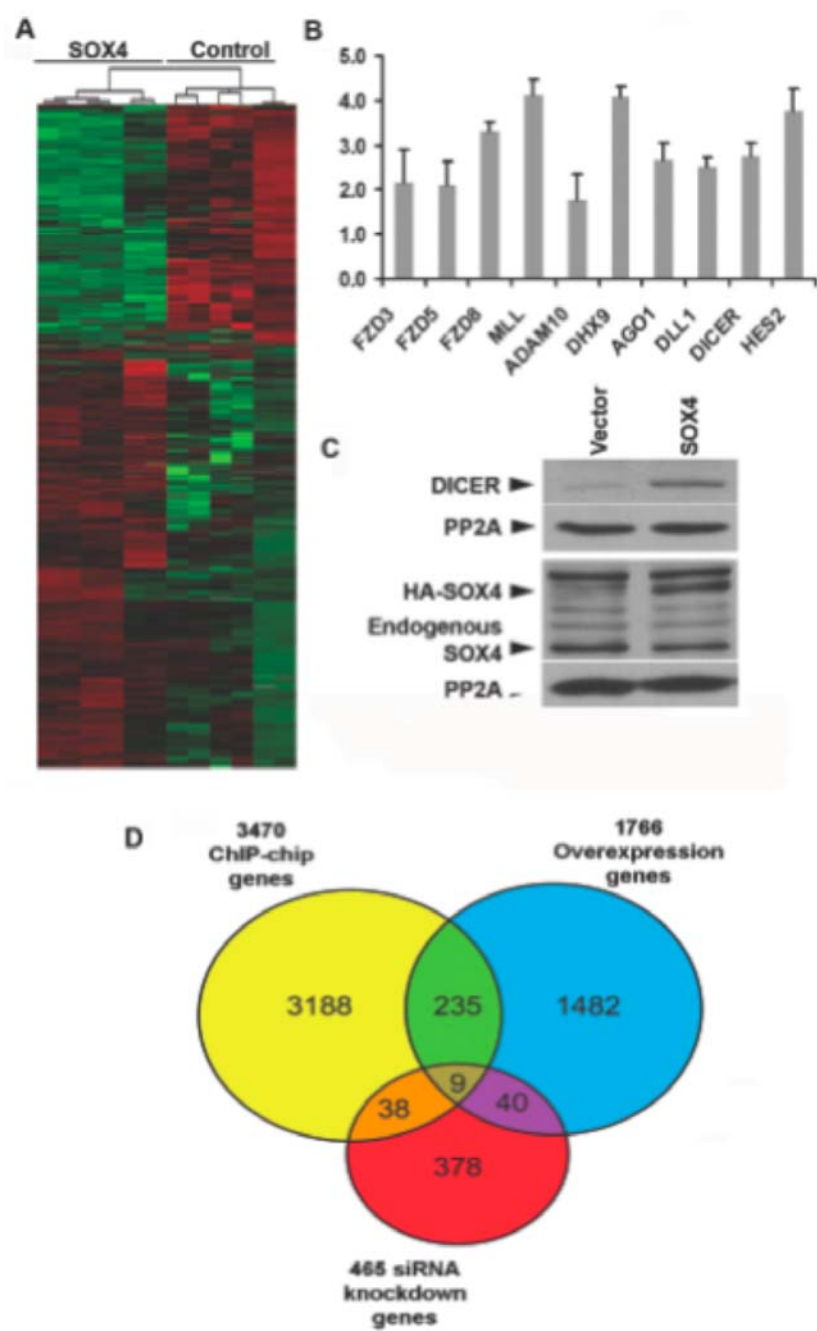


Figure 3: (A) Heat map illustrating Illumina expression data of the 1,766 significant genes as determined by SAM analysis. Red indicates overexpressed and green denotes underexpressed genes. (B) qPCR data of *SOX4* direct target genes after *SOX4* overexpression in LNCaP cells. All ten genes were upregulated over a vector control transfection, similar to values determined by the Illumina array with a p-value less than 0.005 by students T-test. Error bars indicate 1 SD (n = 3 independent biological replicates performed on separate days). (C) *DICER* is regulated by *SOX4* at the protein level. Empty vector or one expressing HA-*SOX4* was transfected into LNCaP cells and immunoblotting performed. *DICER* is upregulated specifically by *SOX4* and not in the control transfection. (D) Venn diagram depicts the overlap between 3,470 ChIP-chip *SOX4* direct target genes, the Illumina expression data set of 1,766 genes, and the Affymetrix expression dataset of 465 genes.

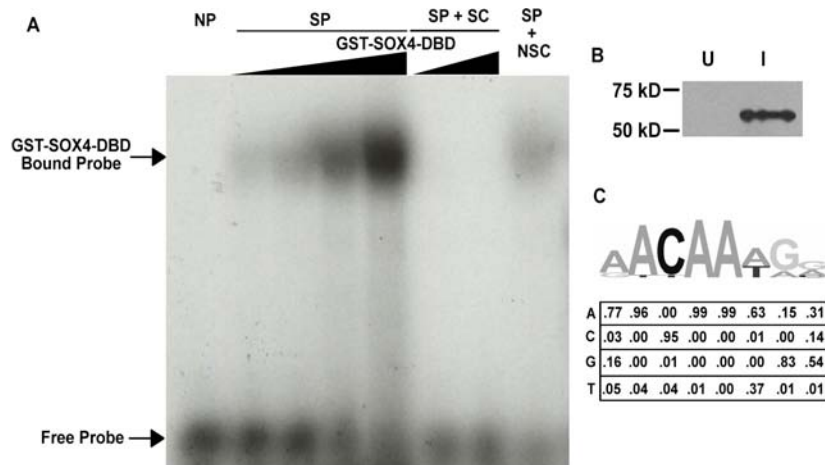


Figure 4: (A) EMSA assay of recombinant GST-SOX4-DBD binding to a known SOX4 binding motif of a 35mer oligo. NP – No protein, SP – specific probe, SC – Specific cold competitor, NSC – non-specific cold competitor. (B) SDS-PAGE gel of GST-SOX4-DBD from an IPTG uninduced (U) or induced (I) cell line. (C) Novel 8mer PWM for SOX4 displayed both graphically and numerically for each base position

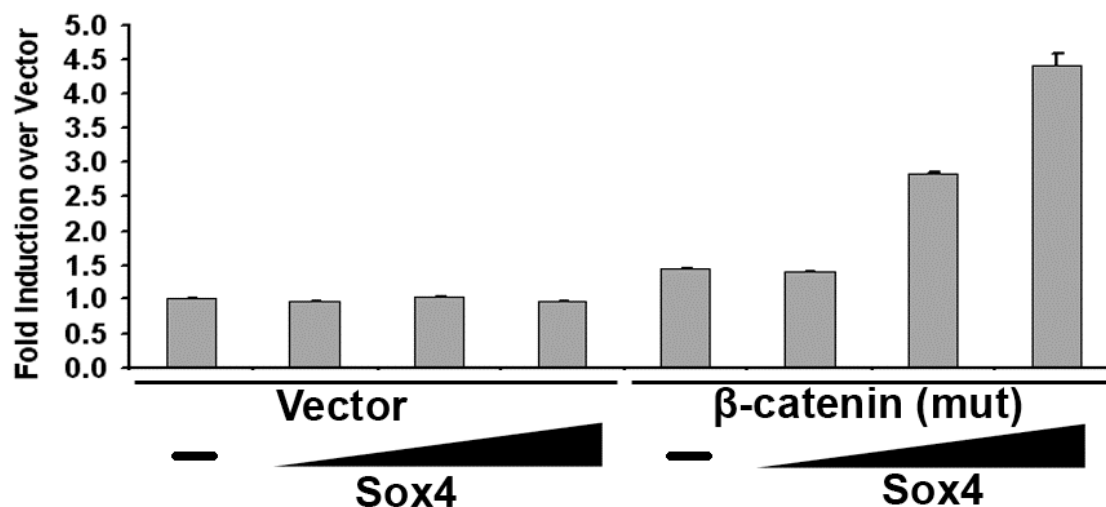


Figure 5: Luciferase assay of LNCaP cells transfected with either a vector control or 100, 200, or 300 ng of a SOX4 expression vector. LNCaP cells were also co-transfected with either a vector control or the β-catenin S33Y constitutively active mutant. All cells were transfected with the TOP flash luciferase reporter and luciferase activity was measured 24 hrs post-transfection. SOX4 does not function alone but instead cooperates with β-catenin to activate the TOP flash reporter in a dose dependent manner.

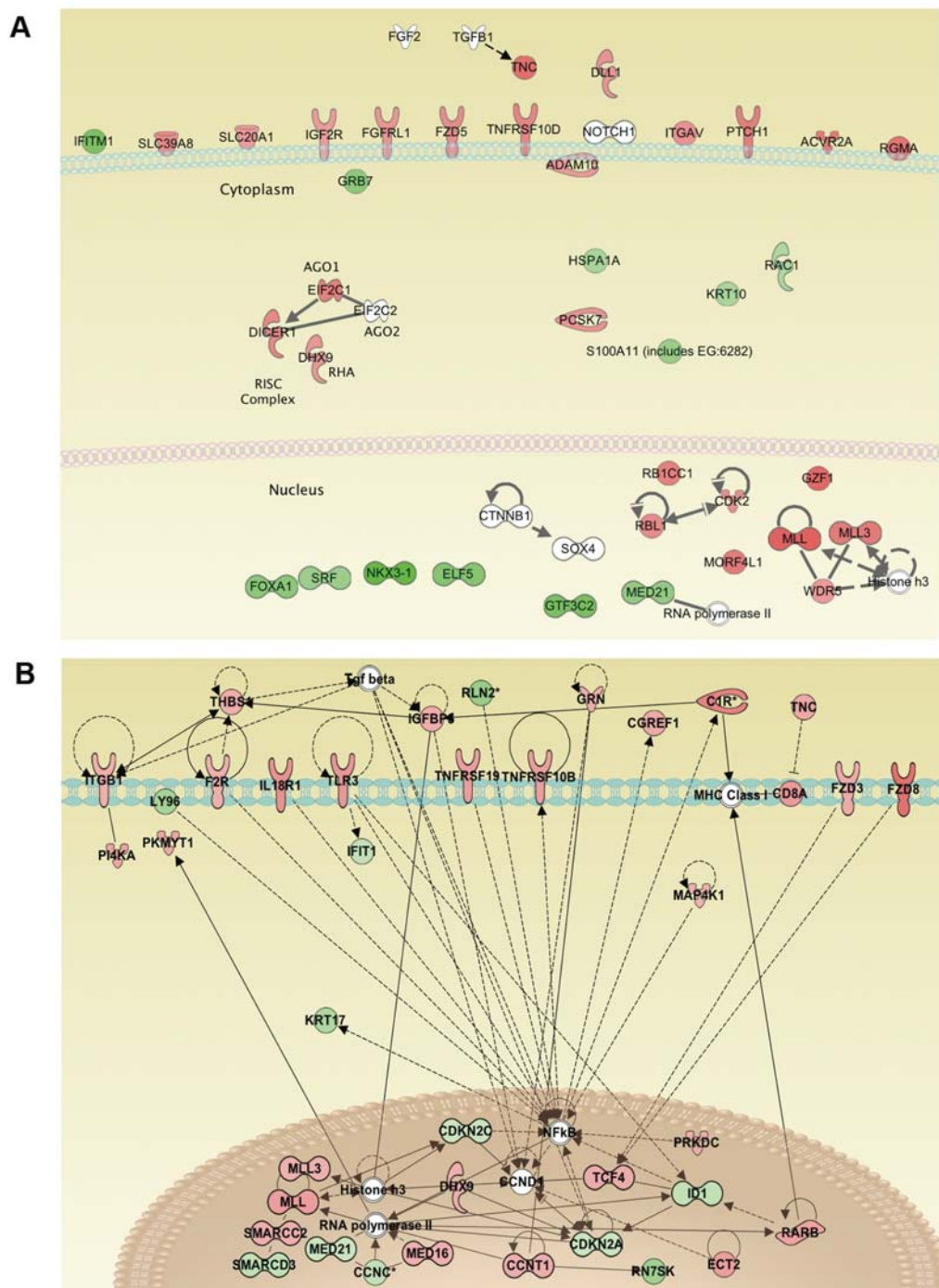


Figure 6: (A) IPA analysis of direct target genes graphically illustrating the cellular location of the *SOX4* transcriptional target genes. *SOX4* regulates a host of nuclear and membrane localized proteins as well as multiple components of the RISC complex. Red indicates target genes upregulated by *SOX4*, green denotes downregulated genes and white represents genes for which no expression change was detected. (B) IPA analysis of Illumina expression genes changed at least 2-fold by SAM analysis. *SOX4* regulatory targets include a host of membrane and nuclear proteins. Red indicates genes upregulated by *SOX4* overexpression and green denotes downregulated genes.

Tables:

<i>Transcription Factor</i>	<i>Family</i>	<i>Benjamini Corrected q-value</i>
E2F4	E2F	1.78E-11
E2F1	E2F	3.06E-11
PAX5	Paired Box	2.07E-10
WHN	Forkhead	2.94E-10
SMAD3	SMAD	1.82E-09
SMAD4	SMAD	3.33E-09
MYC	MYC	6.25E-09
NFKAPPAB	NF-κB	2.95E-08
LEF1/TCF1	LEF	1.12E-06

Table 1: Benjamini corrected q-values for co-occurring transcription factor binding sites.

<i>Entrez ID</i>	<i>Symbol</i>	<i>Microarray Fold Change</i>
196528	ARID2	1.99
2001	ELF5	-2.65
3169	FOXA1	-2.47
2976	GTF3C2	-3.12
64412	GZF1	2.42
84458	LCOR	2.41
4173	MCM4	1.55
58508	MLL3	2.06
10933	MORF4L1	2.07
8031	NCOA4	2.64
4784	NFIX	-2.83
4824	NKX3-1	-4.53
7799	PRDM2	2.48
5933	RBL1	1.80
55509	SNFT	-2.32
6722	SRF	-2.03
54816	SUHW4	-1.93
9412	SURB7	-2.24
9338	TCEAL1	-1.57
7718	ZNF165	1.53
7738	ZNF184	1.66
23528	ZNF281	1.71
30834	ZNRD1	-1.63

Table 2: DAVID analysis identified 23 transcription factors present in our high confidence SOX4 target genes. GO Term: transcription, DNA dependent ($p = 3.7 \times 10^{-18}$).